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Central or Peripheral Delivery of an Adenosine A₁ Receptor Agonist Improves Mechanical Allodynia in a Mouse Model of Painful Diabetic Neuropathy

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Abstract

Diabetic peripheral neuropathy is a common complication of diabetes mellitus, and a significant proportion of individuals suffer debilitating pain that significantly affects their quality of life. Unfortunately, symptomatic treatment options have limited efficacy, and often carry significant risk of systemic adverse effects. Activation of the adenosine A₁ receptor (A₁R) by the analgesic small molecule adenosine has been shown to have antinociceptive benefits in models of inflammatory and neuropathic pain. The current study used a mouse model of painful diabetic neuropathy to determine the effect of diabetes on endogenous adenosine production, and if central or peripheral delivery of adenosine receptor agonists could alleviate signs of mechanical allodynia in diabetic mice. Diabetes was induced using streptozocin in male A/J mice. Mechanical withdrawal thresholds were measured weekly to characterize neuropathy phenotype. Hydrolysis of AMP into adenosine by ectonucleotidases was determined in the dorsal root ganglia (DRG) and spinal cord at 8-weeks post-induction of diabetes. AMP, adenosine and the specific A₁R agonist, N⁶-cyclopentyladenosine (CPA), were administered both centrally (intrathecal) and peripherally (intraplantar) to determine the effect of activation of adenosine receptors on mechanical allodynia in diabetic mice. Eight weeks post-induction, diabetic mice displayed significantly decreased hydrolysis of extracellular AMP in the DRG; at this same time, diabetic mice displayed significantly decreased mechanical withdrawal thresholds compared to nondiabetic controls. Central delivery AMP, adenosine and CPA significantly improved mechanical withdrawal thresholds in diabetic mice. Surprisingly, peripheral delivery of CPA also improved mechanical allodynia in diabetic mice. This study provides new evidence that diabetes significantly affects endogenous AMP hydrolysis, suggesting that altered adenosine production could contribute to the development of painful diabetic neuropathy. Moreover, central and peripheral activation of A₁R significantly improved mechanical sensitivity, warranting further investigation into this important

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Disclosures

The authors have no conflicts of interest to disclose.

antinociceptive pathway as a novel therapeutic option for the treatment of painful diabetic neuropathy.

Introduction

Diabetic peripheral neuropathy is one of the most common and devastating consequences of diabetes mellitus, and occurs in individuals with both type 1 and type 2 diabetes (Duby et al., 2004, Said, 2007, Veves et al., 2008, Van Belle et al., 2011, Callaghan et al., 2012, Gan et al., 2012, McGreevy and Williams, 2012, Lee-Kubli et al., 2014). Up to 30% of patients with diabetic neuropathy experience debilitating pain that significantly affects their quality of life (Krein, 2005, Veves et al., 2008, Ziegler, 2008, Smith and Argoff, 2011). Patients with painful diabetic neuropathy can experience a variety of symptoms, including spontaneous pain, paresthesias (burning, tingling, pins and needles sensations), allodynia (increased sensitivity to a normally innocuous stimuli), or hyperalgesia (heightened sensitivity to an already painful stimulus) (Cole, 2007, Zochodne, 2007, Veves et al., 2008, Smith and Argoff, 2011, Callaghan et al., 2012). Unfortunately, few treatment options exist that provide suitable relief from pain symptoms, and most have variable efficacy or carry significant risk of systemic adverse effects (Cole, 2007, Dworkin et al., 2010, Spallone et al., 2012, Snedecor et al., 2013).

Purinergic signaling is a complex signaling network that has been under investigation for several decades for its role in pain transmission (for review see (Burnstock, 2007)). In the nervous system, ATP is stored in high concentrations in synaptic vesicles and is often co-released with the pro-nociceptive signaling molecules calcitonin gene-related peptide (CGRP) and substance P. Release of ATP results in activation of the ionotropic P2X and metabotropic P2Y receptors, both of which are associated with propagation of pain signals (Sawynok and Liu, 2003, Abbracchio et al., 2009). Termination of ATP-mediated pro-nociceptive signaling is accomplished through the step-wise hydrolysis of ATP into the analgesic small molecule adenosine by specialized enzymes termed ectonucleotidases (Yegutkin, 2008, Longhi et al., 2013).

Previous studies have identified prostatic acid phosphatase (PAP) and ecto-5'-nucleotidase (NT5E) as the main ectonucleotidases responsible for hydrolyzing AMP into adenosine in the DRG and spinal cord (Zylka et al., 2008, Sowa et al., 2010b, Street et al., 2011). These same studies also determined that PAP and NT5E are primarily expressed on isolectin B4 (IB4), P2X₃ positive nonpeptidergic, putative nociceptive neurons, placing them in prime location to modulate nociceptive circuits. Adenosine can activate any of the four known adenosine receptors (A₁, A_{2A}, A_{2B}, A₃), and activation of the A₁ adenosine receptor (A₁R) is associated with antinociceptive outcomes. A₁R is expressed in both the central and peripheral nervous systems, primarily on intrinsic neurons in the dorsal horn of the spinal cord and small- to medium-diameter neurons of the dorsal root ganglia (DRG) (Choca et al., 1988, Sawynok and Liu, 2003, Schulte et al., 2003, Sawynok, 2006). Central activation of A₁R can inhibit synaptic transmission through several mechanisms, including presynaptic inhibition of neurotransmitter release or postsynaptic inhibition of excitatory signals, leading

to decreased transmission of pain sensation (Li and Perl, 1994, Sawynok, 1998, Deuchars et al., 2001).

Studies in rodent pain research have identified adenosine and adenosine receptors as potential therapeutic options to mitigate pain sensation (Lee and Yaksh, 1996, Cui et al., 1997, Sjolund et al., 1998, Fredholm et al., 2001, Bantel et al., 2002, Ulugol et al., 2002, Curros-Criado and Herrero, 2005, Zahn et al., 2007, Horiuchi et al., 2010, Lima et al., 2010, Tian et al., 2010). The current study used a mouse model of painful diabetic neuropathy to determine how diabetes may affect endogenous AMP hydrolysis, and if central or peripheral delivery of adenosine receptor agonists could alleviate signs of mechanical allodynia in diabetic mice. Results show that diabetes significantly decreases the ability of ectonucleotidases to hydrolyze AMP into adenosine in the DRG of diabetic mice. Central delivery of the adenosine receptor agonists, adenosine and AMP, as well as the A₁R-specific agonist N⁶-cyclopentyladenosine (CPA), significantly reversed mechanical sensitivity in diabetic mice. Furthermore, peripheral delivery of CPA significantly improved mechanical allodynia in diabetic mice. Collectively, these data provide evidence that specifically targeting of the A₁R-mediated antinociceptive pathway may be an efficacious treatment option for the management of painful diabetic neuropathy.

Experimental Procedures

Animals

All experiments were approved by the University of Kansas Medical Center Animal Care and Use Protocol. Inbred male A/J mice were obtained from Jackson Laboratories at 7 weeks of age, and were induced with diabetes at 8 weeks of age. All mice were housed in the Laboratory Animal Resources building, had *ad libitum* access food and water, and maintained on a 12 hour light/dark cycle. All mice were sacrificed by 16 weeks of age.

Streptozocin induction of diabetes

Eight week old male A/J mice were injected with Streptozocin (STZ; Sigma, St. Louis, MO) to induce type 1 diabetes. Mice were fasted for 3 hours before and after injections, for a total fasting time of 6 hours. Injections were spread over 2 days, with the first dose (day 1) at 85 mg/kg and the second dose (day 2) at 65 mg/kg. Solutions were made fresh immediately prior to injection and STZ was dissolved in ice cold, filter sterilized 10 mM sodium citrate buffer with 0.9% NaCl at pH 4.5. Control, nondiabetic, mice were injected with sodium citrate buffer only. Mice that did not reach hyperglycemia (blood glucose >230 mg/dL) within one week following initial injections were re-injected with either 85 mg/kg STZ (blood glucose <180 mg/dL) or 65 mg/kg STZ (blood glucose 181–230 mg/dL). Mice that failed to reach hyperglycemia were excluded from the study.

Blood glucose measurements

Weight and blood glucose measurements (glucose diagnostic reagents; Sigma) were collected one week after initial injection and every week thereafter. Mice were fasted for 3 hours prior to collection of blood collection from the tail. Mice were considered diabetic when blood glucose levels were >230 mg/dL.

Behavioral analysis

Prior to behavioral testing, mice were acclimated to the behavior facility and equipment for a minimum of 2 days. On test days, mice were placed in the behavior facility and allowed to acclimate to the environment for at least 30 minutes. Mice were then acclimated on the behavior apparatus for 20 to 30 minutes prior to initiating testing. Mice were placed in individual clear plastic cages on top of a wire mesh grid that allowed access to their hind paws for the duration of the analysis. Mechanical withdrawal thresholds using von Frey monofilaments were measured weekly to track progression of neuropathy phenotype using the up-down method to determine fifty percent withdrawal thresholds (Chaplan et al., 1994). Tests for mechanical sensitivity following intrathecal (i.t.) and intraplantar (i.pl.) injections were performed at 30 min, 1.5 hours, 3 hours and 5 hours following injection; mechanical sensitivity following injection of i.t. purified human PAP (hPAP) was evaluated at 24, 48, 72 and 120 hours post-injection.

Drugs and Drug Administration

Adenosine (Ado), AMP and CPA (direct A₁R agonist) were purchased from Sigma; hPAP was graciously provided by Dr. Mark Zylka (University of North Carolina). 5'-iodotubercidin (ITU; adenosine kinase inhibitor) was purchased from Enzo Life Sciences (Farmingdale, NY). Adenosine (Ado), AMP and CPA were dissolved in 0.9% saline at pH 7.4. AMP was delivered at a concentration of 200 nmol/10 µl (Sowa et al., 2010b); Ado and CPA were delivered at a dose of 10 nmol/10 µl (DeLander and Hopkins, 1987, Lima et al., 2010); hPAP was delivered at a dose of 250 mU/10 µL (Zylka et al., 2008). ITU was co-administered with Ado or AMP at a concentration of 5 nmol/10 µL (Sowa et al., 2010c). Intrathecal injections (hPAP, AMP + ITU, Ado + ITU, CPA) were performed using a 28 gauge, 8 mm insulin syringe (BD Biosciences, San Jose, CA) using the direct lumbar puncture method between L5-L6 (Fairbanks, 2003). Intraplantar (i.pl) injections (AMP + ITU, Ado + ITU, CPA) were performed using a 31 gauge, 1/2" insulin syringe (BD Biosciences) between the distal volar footpads of the right hind paw (Hurt and Zylka, 2012).

Tissue Preparation

Mice were overdosed with inhaled isoflurane, injected with 150 µl of heparin (BD Biosciences) into the ventricle, and then transcardially perfused with 4% paraformaldehyde (in 1 × PBS, pH 7.4). Lumbar spinal cord, dorsal root ganglia (DRG, L4-6) and hind paw skin were dissected, post-fixed in 4% paraformaldehyde for 1 hour, and cryo-protected overnight in 30% sucrose (w/v in 1 × PBS). Tissues were frozen in OTC and stored at -20°C until sectioning. Spinal cord tissue was serially sectioned at 10 µm and DRG were sectioned at 8 µm; tissues were mounted on Suprafrost slides (Fisher Scientific, Chicago, IL) and stored at -20°C until used.

Enzyme Histochemistry

Ectonucleotidase activity was quantified using enzyme histochemistry. Sections of spinal cord or DRG were incubated in trisma-maleate buffer with 8% sucrose (w/v; TMBS; Sigma) at pH 7.4 or pH 5.6 for 30 minutes. Tissues were then incubated in TMBS solution containing 2.4 mM lead nitrate and either 3 mM AMP (DRG, pH 7.4) or 0.3 mM AMP

(DRG, pH 5.6; spinal cord pH 7.4 and pH 5.6) for 2–3 hours. Tissues were developed in 0.5% ammonium sulfide solution, rinsed three times in TMBS, coverslipped and imaged using light microscopy. In the DRG, positive neurons were identified by the presence of a dark brown lead phosphate precipitate within the soma. Soma areas were determined using NIH Image J software by tracing the border of all positive and negative neurons. These data were used to calculate the overall percentage of positive neurons by dividing the number of positive neurons by the overall total number of neurons in each DRG section. Size distribution of positive neurons was determined by binning the soma areas of positive and negative neurons into three bins corresponding to small- ($< 300 \mu\text{m}^2$), medium- ($301\text{--}600 \mu\text{m}^2$), or large-diameter ($601\text{+} \mu\text{m}^2$) neurons. The overall percentage of positive neurons within each bin was determined by dividing the number of positive neurons within each bin by the total number of neurons in that bin. A minimum of 500 neurons per animal were evaluated. Hydrolysis of AMP in the spinal cord was analyzed using NIS Elements software (Nikon Corporation) by placing regions of interest around the medial 1/3 of each band of precipitate in the dorsal horn where the sciatic nerve terminates (Corder et al., 2010). A minimum of 5 dorsal horns per animal were evaluated.

NT5E mRNA quantification using qRT-PCR

RNA was extracted from spinal cord, DRG and skin using TRI Reagent (Sigma) and RNeasy Mini Kit (Qiagen, Valencia, CA). Sample concentration and purity were determined using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Complimentary DNA (cDNA) was synthesized from total RNA using the iScript cDNA synthesis kit (Bio-Rad). qRT-PCR was performed using SsoFast Probes Supermix Kit (Bio-Rad). The primers used were as follows:

NT5E Forward: 5' – CCAGTACCAGGGCACTATCTG – 3'

NT5E Reverse: 5' – TGGCTCGATCAGTCCTTCCA – 3'

GAPDH Forward: 5' – AGGTCGGTGTGAACGGATTTG – 3'

GAPDH Reverse: 5' – TGTAGACCATGAGTTGAGGTCA – 3'

All reactions were performed in triplicate. NT5E mRNA levels (DRG) were normalized to GAPDH. CT values were used to calculate fold change and relative expression levels.

Western Blot Analysis

At sacrifice, spinal cord, DRG and hind paw skin were flash frozen in liquid nitrogen and stored at -80°C until processing. Tissues were sonicated in cell extraction buffer (Invitrogen, Carlsbad, CA) containing 55.5 $\mu\text{L}/\text{mL}$ protease inhibitor cocktail (Sigma, St. Louis, MO), 200 mM Na_3VO_4 and 200 mM NaF for 60 min on ice for protein extraction. Following centrifugation, the protein concentration of the supernatant was determined using the Bradford assay (Bio-Rad, Hercules, CA). Samples were boiled with lane marker reducing sample buffer (Thermo Scientific, Waltham, MA) and stored at -20°C until use. Equal amounts of protein were loaded and separated on a 4–15% gradient Tris-glycine gel (Bio-Rad; 30 mA/gel, 45 min, 4°C), and then transferred to a nitrocellulose membrane (400 mA, 1.5 hours, 4°C). Following incubation with primary and secondary antibodies, bands were visualized using Enhanced Chemiluminescence reagent (ECL; Thermo Scientific).

Membranes were exposed to x-ray film and analyzed using Image J software (NIH). Antibodies include rabbit anti-A₁R (1:500; Thermo Scientific), and goat anti-rabbit (1:5000; Santa Cruz, Dallas, TX).

Membranes were stripped using Restore Plus Western Blot Stripping Buffer (Thermo Scientific), followed by incubation with actin pre-conjugated to HRP (1:10,000; Abcam, Cambridge, MA) overnight at 4°C. Bands were visualized with ECL, membranes exposed to x-ray film and analysis completed using Image J software (NIH).

Statistical analysis

Results were analyzed using the SPSS Statistics 20 software (IBM). Student's t-tests and two-way repeated measures analysis of variance (2-way RM-ANOVA) with Fisher's least significant difference (LSD) posttest analyses were performed, as denoted in the manuscript. A *p*-value less than 0.05 was considered significant. All data are presented as mean ± S.E.M.

Results

Diabetic A/J inbred mice develop mechanical allodynia

Fasting blood glucose and weight measurements were taken weekly following STZ injection. Mice that received STZ had an average fasting blood glucose level of 432.8 ± 18.7 mg/dL and had significantly elevated blood glucose levels as early as one week post-STZ injection, whereas control mice had an average fasting blood glucose level of 101.7 ± 1.9 mg/dL (Fig. 1A). Simultaneously, diabetic mice failed to gain weight compared to their nondiabetic counterparts (Fig. 1B). All mice underwent weekly behavioral testing to quantify changes in hind paw mechanical sensitivity. Beginning 3 weeks post-STZ injections, the withdrawal threshold to mechanical stimuli was significantly decreased in diabetic mice compared to nondiabetic mice (Fig. 1C). Withdrawal thresholds in diabetic mice reached their lowest levels at four weeks postinjection, and remained consistently decreased compared to nondiabetic controls for the duration of the study.

Diabetes alters the ability of ectonucleotidases to hydrolyze AMP in the DRG

Extracellular hydrolysis of AMP is predominantly mediated by PAP and NT5E in the dorsal spinal cord and DRG (Zylka et al., 2008, Sowa et al., 2010b, Street et al., 2011). NT5E is optimally active near physiological pH (pH 7.0), whereas PAP is optimally active at acidic pH (pH 5.6) (Zylka et al., 2008, Sowa et al., 2010b). AMP histochemistry was performed on DRG and spinal cord tissues at pH 7.4 and 5.6 to evaluate the ability of NT5E and PAP, respectively, to hydrolyze AMP in both diabetic and nondiabetic mice. This technique results in the formation of a lead phosphate precipitate upon hydrolysis of the 5'-phosphate group from AMP, which is used to identify neurons capable of hydrolyzing AMP. Examples of AMP hydrolysis in the DRG are shown in Figure 2A. In the DRG of diabetic mice, there was a small but statistically significant decrease in the overall percentage of neurons capable of hydrolyzing AMP at pH 7.4 (Fig. 2B), specifically in neurons that measured within the small-diameter range (<300 μm²; Fig. 2C). At acidic pH, there was no change in the overall percentage of positive neurons (Fig. 2B); however there was a significant decrease in the percentage of positive neurons in the small- (< 300 μm²) and medium-diameter (301–600

μm^2) range (Fig. 2D). Analysis of NT5E mRNA expression in the DRG revealed no significant difference in transcript levels between nondiabetic and diabetic mice at 8-weeks postinduction of diabetes (Fig 2E).

Peripheral neurons from the hind limbs project to characteristic regions in the dorsal horn of the spinal cord and are organized in a somatotopic map such that nerves projecting to the hind paw (the sciatic nerve) terminate in the medial and central regions of the dorsal horn, and nerves innervating more proximal tissues innervate the lateral regions (Corder et al., 2010). Previous studies have shown changes in TMP hydrolysis in the medial portion of the spinal cord in diabetic mice, which develop an insensate neuropathy, compared to nondiabetic mice (Akkina et al., 2001), and it was recently determined that TMPase, the enzyme responsible for hydrolyzing TMP, is the membrane bound form of PAP (Quintero et al., 2007, Zylka et al., 2008). Subsequent work has identified the presence of both PAP and NT5E in the dorsal horn in regions corresponding to central termination patterns of peripheral nociceptive neurons (Zylka et al., 2008, Sowa et al., 2010b). Thus, medial sections of dorsal horn were evaluated to determine the effect of diabetes on AMP hydrolysis in the sciatic nerve of diabetic mice compared to nondiabetic mice. Examples of AMP hydrolysis at pH 7.4 and 5.6 in the dorsal horn of nondiabetic and diabetic mice are shown in Figure 3A. Assessment of enzyme reactivity in the medial portions of the dorsal horn revealed no significant differences in staining density between nondiabetic and diabetic mice at physiological or acidic pH (Fig. 3B).

Diabetes does not alter A₁R expression in the spinal cord or DRG

The antinociceptive effects of adenosine are attributed to activation of A₁R, and this receptor is highly expressed in the dorsal horn of the spinal cord and DRG in close proximity to peripheral pain sensing neurons (Choca et al., 1988, Schulte et al., 2003). To determine the effect of diabetes on A₁R expression, Western blot analysis was employed to evaluate protein expression levels of this receptor in both the spinal cord (Fig. 4A) and DRG (Fig. 4B). No significant differences in expression were seen between diabetic and nondiabetic mice in either tissue; however, in the spinal cord A₁R expression was trending towards a significant decrease ($p = 0.056$) in diabetic mice compared to nondiabetic mice.

AMP hydrolysis in the spinal cord of diabetic mice improves mechanical hyperalgesia

In light of the observation that diabetes affects AMP hydrolysis, a series of *in vivo* experiments were performed in an attempt to activate the endogenous A₁R-mediated antinociceptive pathway to determine if this could improve signs of mechanical hyperalgesia in diabetic mice. Previous studies indicate that a single dose of i.t. hPAP provided long lasting (>3 days) antinociceptive effects in mice that had either inflammatory or neuropathic pain (Zylka et al., 2008, Sowa et al., 2009, Sowa et al., 2010a). To determine if hPAP had similar efficacy in painful diabetic neuropathy, a single injection of i.t. hPAP (250 mU/10 μl) was administered to diabetic mice. Diabetic mice treated with i.t. hPAP displayed a significant reversal of their mechanical sensitivity at 24 hours post-injection compared to baseline values ($p = 0.02$, Fig. 5A); however, this was not significantly different than diabetic vehicle treated mice at 24 hours.

Subsequently, i.t. AMP (200 nmol/10 μ l) was administered to take advantage of the endogenous hydrolytic capacity of ectonucleotidases to generate adenosine from AMP to determine if saturation of these enzymes with substrate was sufficient to activate antinociceptive pathways. To increase the duration of time that adenosine is present in the extracellular space, the nucleotide transporter inhibitor, 5'-iodotubercidin (ITU; 5 nmol/10 μ l), was co-administered with AMP. When given alone, neither AMP nor ITU (nor adenosine; see below) produced any changes in mechanical sensitivity in diabetic mice (data not shown). However, when AMP was co-administered with ITU (i.t. AMP + ITU) diabetic mice showed a significant, but transient, reversal of their mechanical sensitivity at 30 min ($p = 0.01$) compared to pre-treatment withdrawal thresholds (Figs. 5B). The withdrawal threshold of AMP + ITU treated diabetic mice was also significantly different than diabetic vehicle treated mice after 30 min ($p = 0.002$).

Direct activation of spinal adenosine receptors significantly improves mechanical allodynia in diabetic mice

The observation that i.t. administration of AMP could improve mechanical allodynia in diabetic mice provided indirect evidence that adenosine receptors in diabetic mice were able to signal properly. Thus, interventions that directly targeted the adenosine receptors were performed in an effort to improve the duration of efficacy observed following delivery of i.t. AMP + ITU. Intrathecal adenosine (10 nmol/10 μ l) was delivered to bypass enzyme-mediated hydrolysis of AMP and directly activate adenosine receptors, rather than indirectly targeting the receptors via substrate hydrolysis. Adenosine was co-administered with ITU (Ado + ITU) to increase the length of time that adenosine was present in the extracellular space. Diabetic mice that received Ado + ITU displayed a significant increase in mechanical withdrawal threshold for at least 5 hours compared to baseline ($p < 0.01$), as well as compared to diabetic vehicle treated mice ($p < 0.05$); at this time withdrawal thresholds had not yet returned to pre-treatment levels (Fig. 6A).

To test whether the observed antinociceptive benefits following i.t. administration of AMP + ITU and Ado + ITU in diabetic mice were mediated in part by activation of A₁R, the specific A₁R agonist and stable adenosine analog, CPA (10 nmol/10 μ l), was administered to specifically activate this receptor. Diabetic mice that received i.t. CPA displayed a significant increase in mechanical withdrawal thresholds compared to baseline and diabetic vehicle treated mice, lasting up to 3 hours ($p < 0.01$; Fig. 6B).

Peripheral delivery of an A₁R agonist reduces mechanical hypersensitivity

Use of topical analgesics, including topical capsaicin and topical lidocaine, have shown efficacy for the treatment of neuropathic pain conditions such as painful diabetic neuropathy, post-herpetic neuralgia and human immunodeficiency virus-induced neuropathy (Simpson et al., 2008, Baron et al., 2009, Irving et al., 2011, Argoff, 2013). Thus, peripheral delivery of adenosine receptor agonists were administered to diabetic and nondiabetic mice to determine if activation of adenosine receptors from the periphery could provide an efficacious alternative for the treatment of painful diabetic neuropathy. Protein levels of A₁R in the hind paw of diabetic and nondiabetic mice were detected using qRT-PCR and Western blot analysis (data not shown) and no differences in mRNA or protein expression

were identified. Additionally NT5E, but not PAP, has been detected in the hind paw skin of mice (Sowa et al., 2010b). For this reason, i.pl. AMP + ITU was administered to determine if endogenous enzymatic hydrolysis of AMP in the hind paw was sufficient to activate peripheral adenosine receptors; however, this was not effective at alleviating mechanical allodynia in diabetic mice (data not shown).

Subsequent administration of i.pl. Ado + ITU also failed to alleviate mechanical sensitivity in diabetic mice, who remained consistently allodynic throughout the duration of the experiment (Fig 7A). Additionally, diabetic mice treated with i.pl. Ado + ITU displayed mechanical withdrawal thresholds that were significantly decreased compared to diabetic vehicle treated mice at 30 min and 1.5 hours following injection ($p < 0.05$). In contrast, i.pl. administration of CPA was able to transiently reverse mechanical sensitivity in diabetic mice for up to 3 hours compared to baseline ($p < 0.01$) and diabetic vehicle treated mice ($p < 0.01$; Fig. 7B).

Discussion

Pain affects a significant proportion of individuals who develop diabetic peripheral neuropathy. Although many mechanisms have been identified as potential underlying causes, few have resulted in the development of successful therapeutic treatment options, emphasizing the need for development of novel therapies for mitigating pain sensation. This study highlights the fact that while endogenous adenosinergic signaling may be slightly altered due to diabetes, activation of the A₁R-mediated antinociceptive pathway can alleviate pain associated with diabetic neuropathy, specifically mechanical sensitivity. Importantly, we showed both central and peripheral methods of activation that has significant implications when translating this body of work to human subjects. To our knowledge, this is the first study to demonstrate an effect of diabetes on the hydrolytic capacity of ectonucleotidases. Additionally, this is one of the first studies to demonstrate peripherally-mediated antinociception in a rodent model painful diabetic neuropathy.

Diabetes affects AMP hydrolysis in the peripheral nervous system

Under pathological conditions, nucleotides, such as ATP, can be released into the extracellular space from vesicles at nerve synapses, and also following tissue injury from damaged and dying cells. These nucleotides can then go on to activate a variety of receptors, including pro-nociceptive P2X and P2Y receptors, resulting in pain sensation and nociceptive neurotransmission (Burnstock, 2007, 2008, Yegutkin, 2008). Ectonucleotidases function to terminate this nociceptive signaling by hydrolyzing pronociceptive nucleotides (ATP, ADP and AMP) into the antinociceptive small molecule adenosine (Zimmermann, 1998, Goding, 2000, Yegutkin, 2008). Recently, Zylka and colleagues demonstrated the important role that PAP and NT5E play in modulating mechanical allodynia and thermal hyperalgesia in rodent models of inflammatory and neuropathic pain (Zylka et al., 2008, Sowa et al., 2010b).

In the DRG, PAP and NT5E have been shown to co-localize extensively with markers that are used to identify nonpeptidergic, putative nociceptive neurons such as P2X₃, IB4 and *Mrgprd* (Zylka et al., 2008, Sowa et al., 2010b, Taylor-Blake and Zylka, 2010). Nociceptive

neurons are also identified based on size, and fall within the small- (unmyelinated C-fibers) to medium- (thinly myelinated A δ -fibers) diameter range of neuron size. Our studies reveal that this specific neuronal population that is affected by diabetes, resulting in decreased hydrolysis of AMP in the DRG of diabetic mice. Although this decrease in AMP hydrolysis is subtle, it is nonetheless significant and could have a major physiological impact, such as altered mechanical sensitivity. Indeed, the time at which enzyme activity was measured in this model correlates with significantly reduced mechanical withdrawal thresholds in diabetic mice, suggesting a link between reduced AMP hydrolysis (and subsequent adenosine production) and mechanical sensitivity in the setting of diabetes. Transcript levels of NT5E mRNA were not significantly different between nondiabetic and diabetic mice in the DRG at 8-weeks post-induction of diabetes, suggesting that diabetes does not affect protein (enzyme) expression, but rather interferes with protein function as evidenced by decreased hydrolysis of AMP in the DRG at both pH 7.4 and 5.6 in diabetic mice compared to nondiabetic mice. Furthermore, protein levels of A $_1$ R in the DRG were not affected by diabetes, suggesting that it is decreased adenosine production, leading to decreased receptor activation, rather than changes in receptor expression, that contribute to the observed phenotype.

Endogenous adenosine production transiently improves mechanical hyperalgesia

Both PAP and NT5E are expressed in lamina II of the spinal cord where the central projections of peripheral nociceptive nerve fibers terminate, placing them in prime location to modulate nociceptive signaling (Zylka et al., 2008, Sowa et al., 2010b, Taylor-Blake and Zylka, 2010). In this study, diabetes resulted in decreased enzymatic hydrolysis of AMP in the peripheral nervous system, but not the central nervous system, providing yet another piece of evidence that diabetes primarily affects the peripheral branches of afferent nociceptive neurons. Although no changes in AMP hydrolysis were detected in the spinal cord, a near significant decrease in protein levels of A $_1$ R were detected in diabetic mice. Collectively, decreased adenosine production in the periphery coupled with changes in protein expression of A $_1$ R centrally could lead to an overall significant decrease in activation of this physiologically important antinociceptive pathway, which could manifest as increased mechanical sensitivity. Future studies should explore signaling pathways downstream of A $_1$ R to determine the effects of diabetes on A $_1$ R-mediated signaling.

Previous studies indicate that a single injection of i.t. hPAP has antinociceptive benefits which can last up to three days in models of inflammatory and neuropathic pain, and these results were dependent upon activation of A $_1$ R (Zylka et al., 2008, Sowa et al., 2010a, Hurt and Zylka, 2012). Given that i.t. hPAP was successful in alleviating thermal hyperalgesia in a model of neuropathic pain, and the observation that diabetes affects the peripheral nervous system, we sought to determine if this intervention could successfully improve mechanical hyperalgesia in our rodent model of painful diabetic neuropathy. Unfortunately, no significant improvements in mechanical sensitivity were observed in diabetic hPAP-treated mice compared to diabetic-vehicle treated mice. This lack of efficacy could be due to elements of the disease process interfering with intrinsic enzyme activity, as was seen using AMP histochemistry in the periphery, thus preventing maximal activation of A $_1$ R. Alternatively, it is possible that the peak antinociceptive effect of i.t. hPAP occurred prior to

the 24 hour time point at which the first assessment of mechanical sensitivity was performed (based on previously referenced experiments), thereby preventing our ability to observe a significant antinociceptive effect following i.t. administration of hPAP. Despite the lack of effect seen with this approach, central delivery of AMP resulted in a transient but significant improvement in mechanical withdrawal thresholds, suggesting that endogenous hydrolysis of AMP and subsequent production of adenosine was sufficient to activate antinociceptive pathways in diabetic mice.

Central activation of A₁R modulates nociception in diabetic mice

Although AMP hydrolysis in the spinal cord was sufficient to activate antinociceptive pathways and improve mechanical allodynia, the duration of this effect was relatively short-lasting (< 1.5 hours); however, administration of i.t. Ado + ITU resulted in long-lasting (> 5 hours) improvements in mechanical sensitivity. This provides strong evidence that direct activation of adenosine receptors by endogenous ligands, rather than indirect activation through enzyme-mediated hydrolysis of substrates, is a more efficacious route for development of treatment interventions. Because adenosine can act at any of the four adenosine receptors, the stable adenosine analog and direct activator of A₁R, CPA, was used to provide evidence that the behavioral outcomes observed in response to i.t. adenosine could be mediated through activation of A₁R. This study shows that activation of A₁R by CPA resulted in a reversal in mechanical sensitivity similar to that seen following i.t. administration of Ado + ITU, which lasted several hours as compared to indirect activation via hydrolysis of AMP by ectonucleotidases. Collectively, these data show that both adenosine and CPA were both capable of alleviating mechanical sensitivity, and these results are likely mediated through activation of A₁R. Future studies should include the use of A₁R specific antagonists to confirm that the behavioral outcomes observed in this study in response to i.t. AMP + ITU and i.t. Ado + ITU were indeed mediated by activation of A₁R.

Indeed, several studies have shown that the antinociceptive properties of adenosine are driven by activation of A₁R through the use of selective agonists and antagonists (Cui et al., 1997, Sjolund et al., 1998, Bantel et al., 2002, Curros-Criado and Herrero, 2005, Horiuchi et al., 2010, Lima et al., 2010, Sowa et al., 2010a, Tian et al., 2010, Fredholm et al., 2011). Furthermore, Tian et al. report that A₁R is under tonic activation by adenosine, which may contribute to setting a “physiological nociceptive threshold” (Tian et al., 2010). Disturbances in this balance, as suggested here by decreased enzymatic activity leading to decreased adenosine production, as well as alterations in protein levels of A₁R, could shift the threshold from a normal pain-free setting to a pro-nociceptive condition which could influence pain states under chronic disease conditions. Additionally, Guieu et al. report that patients with chronic neuropathic pain have lower circulating levels of adenosine in their blood and cerebrospinal fluid, and this loss of tonic activation of antinociceptive pathways could contribute to increased pain sensation in chronic disease settings (Guieu et al., 1996). Therapies that target this endogenous system and aim to correct this imbalance may provide a suitable alternative for mitigating pain sensation in painful diabetic neuropathy.

Peripheral activation of A₁R is antinociceptive

Current treatment options for the management of painful diabetic neuropathy are primarily limited to oral-acting substances for symptomatic relief. Unfortunately, these oral treatment options come with significant risk of systemic adverse effects that often limits their efficacy and contributes to patient non-compliance (Dworkin et al., 2010, Smith and Argoff, 2011, Callaghan et al., 2012). Topical treatment modalities have gained attention recently as alternatives to traditional oral medications (Sawynok, 2003, Argoff, 2013), and have shown success for the management of pain in several conditions such as post-herpetic neuralgia, HIV-induced neuropathy and, albeit limited, painful diabetic neuropathy (Simpson et al., 2008, Baron et al., 2009, Dworkin et al., 2010, Irving et al., 2011). Thus, we sought to determine if peripheral activation of A₁R could result in improvement in mechanical sensitivity in our model of painful diabetic neuropathy as an alternative to intrathecal administration and central activation of this antinociceptive pathway.

Peripheral administration of adenosine can have different effects depending on the receptor subtype activated. For example, direct administration of adenosine to the hind paw of rats results in cutaneous hyperalgesia and decreased paw withdrawal thresholds, a result likely mediated through activation of the A₂ adenosine receptor and increased cAMP production (Taiwo and Levine, 1990). Use of specific agonists for the different adenosine receptors helped elucidate the differential effects of peripheral activation of these receptors. Peripheral administration of CPA to the rat hind paw decreased prostaglandin E₂-mediated inflammatory pain (Taiwo and Levine, 1990, Aley et al., 1995), and administration of the A₁R agonist, R-phenylisopropyl-adenosine (RPIA), decreased formalin-induced nociceptive behaviors (Karlsten et al., 1992). In contrast, administration of the A₂ adenosine receptor agonist, 2-(2-aminoethylamino)-carbonylphenyl-ethylamino-adenosine (APEC), resulted increased nociceptive behaviors in response to formalin injection in the rat hind paw (Karlsten et al., 1992). Activation of the A₃ adenosine receptor in the periphery has also been implicated in mediating the algescic effects of adenosine, primarily through stimulatory effects on mast cells resulting in the release of histamine and 5-hydroxytryptamine (Sawynok et al., 1997).

Results of this study suggest peripheral administration of adenosine has an algescic effect as evidenced by consistently decreased mechanical withdrawal thresholds in diabetic mice treated with i.pl. Ado + ITU compared to diabetic vehicle treated mice. With this limited information the authors are unable to speculate as to the mechanism contributing to this observation; however, the results of these studies fall in line with previously published literature as to the pain-inducing effects of peripheral administration of adenosine. To our surprise, peripheral administration of CPA was able to improve mechanical withdrawal thresholds in diabetic mice for several hours, suggesting that specific activation of peripheral A₁R-mediated antinociceptive pathways may be effective at alleviating signs of mechanical hyperalgesia associated with painful diabetic neuropathy. This warrants further investigation into the efficacy of topical analgesics that activate A₁R for treating painful diabetic neuropathy and other neuropathic pain conditions.

Concluding remarks

This study utilized a rodent model of painful diabetic neuropathy to evaluate the effect of diabetes on the generation of adenosine. Subsequently, behavioral studies were primarily focused on determining the efficacy of adenosine receptor-mediated antinociceptive pathway activation on alleviating mechanical allodynia in diabetic mice. Results show that direct central activation of adenosine receptors by adenosine or CPA provided long-lasting antinociceptive effects, whereas indirect activation of adenosine receptors by AMP through hydrolysis by ectonucleotidases (such as PAP and NT5E) is less efficacious. Our study provides new evidence that peripheral activation of A₁R by CPA can temporarily reverse mechanical sensitivity in painful diabetic neuropathy. This result highlights the importance of this antinociceptive pathway in modulating pain sensation under normal and pathological conditions, and could provide a novel therapeutic treatment option for patients suffering from painful diabetic neuropathy.

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Highlights

- Male A/J mice develop mechanical allodynia following induction of diabetes
- Diabetes decreases AMP hydrolysis in the DRG in small and medium diameter neurons
- Central delivery of A₁R agonists improves mechanical allodynia in diabetic mice
- Peripheral delivery of an A₁R specific agonist improves mechanical allodynia

Figure 1. Diabetic mice develop hyperglycemia and mechanical allodynia

A) Blood glucose measurements of diabetic (DB) mice were significantly elevated at all time points beginning 1 week post-STZ injection ($p < 0.0001$ vs. nondiabetic [ND] at all time points; 2-way RM-ANOVA and LSD posttest). B) Diabetic mice failed to gain weight, unlike their ND counterparts ($p < 0.0001$ vs. ND at all time points; 2-way RM-ANOVA and LSD posttest). C) Diabetic mice displayed a significantly decreased mechanical withdrawal threshold beginning 3 weeks post-STZ injection that persisted throughout the duration of the

study (** $p < 0.001$, **** $p < 0.0001$ vs. ND; 2-way RMANOVA and LSD posttest). $n = 28$ – 33 mice per group.

Figure 2. AMP hydrolysis is decreased in the DRG of diabetic mice

A) Representative images of AMP histochemistry performed in DRG sections from nondiabetic and diabetic mice at pH 7.4 and 5.6. Arrowheads indicate positive neurons; asterisks indicate negative neurons. B) The overall percentage of neurons displaying evidence of hydrolyzed AMP at pH 7.4 was significantly decreased in diabetic (DB) mice, compared to nondiabetic (ND) mice ($*p < 0.05$ vs. ND, t-test). There was no significant difference in the overall percentage of neurons capable of hydrolyzing AMP at pH 5.6 ($p > 0.05$ vs. ND, t-test). C) The percentage of positive neurons was significantly decreased only within small-diameter ($< 300 \mu\text{m}^2$) neurons at pH 7.4 ($*p < 0.05$ vs. ND, 2-way RM-ANOVA and LSD posttest). D) At pH 5.6, the percentage of positive neurons was significantly decreased within both the small- ($< 300 \mu\text{m}^2$) and medium-diameter (301–600 μm^2) neurons ($*p < 0.05$ vs. ND, 2-way RM-ANOVA and LSD posttest). $n = 7$ mice per group. E) Analysis of NT5E mRNA expression at 8-weeks post induction of diabetes did not reveal any significant differences in mRNA expression levels between ND and DB mice ($p > 0.05$ vs. ND, t-test). $n = 6$ mice per group.

Figure 3. Diabetes does not affect AMP hydrolysis in the dorsal horn of the lumbar spinal cord
 A) Representative images of AMP histochemistry in dorsal horn sections from nondiabetic (ND) and diabetic (DB) mice at pH 7.4 and pH 5.6. B) No significant difference in AMP hydrolysis was observed between ND and DB mice at pH 7.4 or at pH 5.6 in the medial portion of the dorsal horn ($p > 0.05$ vs. ND, t-test). $n = 5-7$ mice per group.

Figure 4. Protein levels of A₁R are not affected by diabetes in the spinal cord or DRG

A) Following eight weeks of diabetes, A₁R protein levels in the spinal cord were not significantly different in diabetic (DB) compared to nondiabetic (ND) mice ($p > 0.05$ vs. ND; t-test). B) A₁R protein expression was not significantly different between ND and DB mice in the DRG ($p > 0.05$ vs. ND; t-test). $n = 7-9$ mice per group.

Figure 5. Supplementation of i.t. hPAP and AMP + ITU transiently improves mechanical sensitivity in diabetic mice

A) Diabetic (DB) mice administered i.t. hPAP (250 mU/10 μ l) showed a small but significant improvement in mechanical withdrawal thresholds compared to baseline levels after 24 hours ($\# p < 0.05$ vs. DB hPAP, 2-way RM-ANOVA with LSD posttest); however this was not significantly different from diabetic vehicle treated mice at 24 hours ($p > 0.05$ vs. DB Veh, 2-way RM-ANOVA with LSD posttest). $n = 9$ –10 mice per group. B) Diabetic mice administered i.t. AMP + ITU (200 nmol AMP/10 μ l, 5 nmol ITU/10 μ l) showed a significant reversal in their mechanical sensitivity at 30 min following injection compared to

baseline ($## p = 0.01$ vs. DB AMP + ITU, 2-way RM-ANOVA with LSD posttest) and compared to DB vehicle treated mice ($**p < 0.01$ vs. DB Veh, 2-way RM-ANOVA with LSD posttest), which returned to pre-injection withdrawal thresholds after 1.5 hours. $n = 9-10$ mice per group.

Figure 6. Central delivery of Ado + ITU or CPA provides long-lasting anti-allodynic effects in diabetic mice

A) Diabetic (DB) mice that received i.t. Ado + ITU (Ado, 10 nmol/10 μ l; ITU, 5 nmol/10 μ l) showed a significant reversal of mechanical sensitivity compared to baseline (## $p < 0.01$ vs. DB Ado + ITU, 2-way RM-ANOVA with LSD posttest) and diabetic vehicle treated mice (* $p < 0.05$ vs. DB Veh, 2-way RM-ANOVA with LSD posttest) that lasted over 5 hours, at which time withdrawal thresholds had not yet returned to baseline levels. $n = 8$ mice per group. B) Intrathecal CPA (10 nmol/10 μ l) also significantly reversed mechanical withdrawal thresholds in diabetic mice compared to baseline (## $p < 0.01$ vs. DB CPA, 2-

way RM-ANOVA with LSD posttest) and diabetic vehicle treated mice (** $p < 0.01$ vs. DB Veh, 2-way RM-ANOVA with LSD posttest) for up to 3 hours. $n = 7 - 9$ mice per group.

Figure 7. Peripheral delivery of CPA, but not Ado, significantly improved mechanical allodynia in diabetic mice

A) Diabetic (DB) mice treated with i.pl. Ado + ITU (Ado 10 nmol/10 μ l; ITU 5 nmol/10 μ l) remained consistently allodynic throughout the duration of the experiment, and had significantly reduced withdrawal thresholds compared to DB vehicle treated mice at 30 min and 1.5 hours post-treatment (* $p < 0.05$ vs. DB Veh; 2-way RM-ANOVA with LSD posttest). $n = 8$ mice per group. B) Intraplantar administration of CPA (10 nmol/10 μ l) significantly reversed mechanical allodynia in diabetic mice compared to baseline (### $p <$

0.001 vs. DB CPA, 2-way RM-ANOVA with LSD posttest) and compared to vehicle treated diabetic mice (** $p < 0.01$ vs DB Veh, 2- way RM-ANOVA with LSD posttest) which lasted up to 3 hours following injections. $n = 4 - 5$ mice per group.